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MICROBIOLOGICAL POLLUTION INDICATORS. STATE OF THE ART. 1603 - BIOLOGICAL IDENTIFICATION

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1603 - BIOLOGICAL IDENTIFICATION - MICROBIOLOGICAL POLLUTION INDICATORS

by

Francis T. Brezenski

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1603 - Biological Identification - Microbiological Pollution Indicators

Coliform Bacteria

An excellent compilation of research reports on developments in sanitary bacteriology have been published under the heading of "Sanitary Significance of Fecal Coliforms In The Environment".[1] Since the historical developments have been exhaustively reviewed - there is no need to present another review. A discussion of the ensuing developments and philosophy will suffice to develop the present microbial indicator systems routinely used to detect fecal contamination in bodies of water. Major historical developments are interjected solely to maintain continuity of the subject matter.

The coliform group of bacteria have been utilized as indicators of water quality for many years. The Bacillus coli as Escherich described has been considered characteristic of human feces.[2] The presence of these organisms indicated a potential health hazard because of their association with the enteric pathogenic microorganisms. Since that time, investigators broadened this to include a whole range of organisms into what is currently known as the coliform group. Additional organisms were placed into this group according to specific biochemical reactions. Investigators examined biochemical reactions which would more effectively differentiate those species or strains isolated from the excrement of warm blooded animals from

saprophytic strains which were associated with soil and plant materials. A significant application was provided by Parr in 1938[3] when he suggested the use of a combination of four reactions which at that time appeared to be most specific in separating non-fecal and fecal coliforms. He compounded the indole, methyl red, Voges-Proskauer and citrate tests into a series called the "IMViC Test". The series of four tests give 16 possible combinations. The interpretation of these combinations was described by Parr as follows:

Escherichia group: comprising IMViC types ++--, +--- and -+--, which he considered to be of fecal origin.

Aerobacter group: comprising IMViC types --++, --+- and ---+, which represent the types found in soil.

Intermediate group: consisting of the remaining 10 possible IMViC types.

When a statistically significant number of strains from a single source were examined by this method, good results were obtained with respect to the fecal or non-fecal classification of the coliform group. However, discrepancies arose in the relationship between sources (fecal, soil and vegetation) with IMViC types when only a few strains were examined from different sources. Consequently, interpretation of such data becomes difficult and reliability decreases. Laboratory data has also demonstrated that strains in the natural environment may display changes in 2 of the 4 reactions. For example, laboratory experiements have changed indole-positive to indole-negative strains and have caused the reversion of positive Voges-Proskauer strains.

Investigators not satisfied with the IMViC classification system, continued work to better differentiate between fecal and non-fecal types. Eijkman[4] proposed the elevated temperature test for the separation of fecal and non-fecal types. The test relied upon the premise that fecal coliforms produced gas from glucose at 46°C while non-fecal coliforms failed to grow. There was some question as to the sensitivity and specificity of the test at this temperature. A number of studies on the selectivity and sensitivity of the elevated temperature test have resolved 44.5°C as the most acceptable temperature of incubation.[5,6] Geldreich, et. al., used the elevated temperature test to study coliform strains isolated from feces, soil, vegetation, insects and fish.[7,8,9,10] With fecal samples, they found good correlation of the elevated temperature test with the Parr fecal group classified by IMViC reactions and that soil coliforms correlated well with the Parr non-fecal group. The test, however, is not a perfect one and the establishment of 44.5°C represents a compromise between acceptable sensitivity and specificity for the fecal types. At this temperature range (44.5°C $\pm$ 0.5) a small number of non-fecal coliforms are included. The EC or elevated temperature test medium can only be used as a confirmatory procedure when subculturing is carried out from non-selective media. Direct inoculation of sample into EC medium without prior enrichment will generally produce lower fecal coliform results. Consequently, the EC medium cannot be used <u>directly</u> to confirm colonies grown on membrane filters.

When the EC procedure was established, the basic premise used in the evaluation of the results was that all coliforms found in the feces of warm blooded animals must be realized as fecal strains while those isolated from

non-polluted soils (as far as could be determined) were non-fecal coliform strains. The difficulty arises in being able to establish the fact that contamination of the soil with fecal material did not occur at some time or other. It is possible that some of the soil samples used in the development of this test and considered as non-polluted had actually received small amounts of fecal contamination from domestic or wild animals. In view of the lack of availability of a positively known non-polluted soil sample, such specimens had to be considered as "reference samples" for the fecal and non-fecal definitions. At the present time a more adequate or definitive source description cannot be given. However, results of many investigations have shown this premise to be a workable one and the question of source reference samples is academic and does not negate its use in pollution studies. The technique is adequate for pollution detection and separation of fecal and non-fecal groups.

The specificity of the fecal coliform elevated temperature test procedure has been questioned and reported in the literature.[11,12,13] Such opposition has refused to accept the more current philosophy that any coliform derived from feces and producing gas from lactose at 44.5°C - regardless of IMViC type is a true fecal coliform. The elevated temperature test therefore will include *E. coli* and other IMViC types growing and producing gas from lactose at 44.5°C. On the other hand, the opposing minority provides a narrow definition for the fecal coliform group which includes the classical *E. coli* types only. Based on this philosophy, results of comparative studies

have shown discrepancies. For example, Slanetz et. al.[12,13] reported that the EC (elevated temperature test) MPN procedure gave significantly higher values than did the E. coli MPN technic. E. coli could be detected in only approximately 20% of the EC-MPN positive cultures. It was surmised that the effect was due to the presence or overgrowth by other coliform biotypes in the EC medium. The investigators concluded that such results raise a question as to the validity of the EC-MPN determinations for fecal coliforms in sea water especially if one does not accept the basic tenet that all types of coliforms growing in the EC medium at 44.5°C are generally of fecal origin. The type incubator used and temperature of incubation was also questioned.

Recommended procedures for fecal coliforms in sea water and shellfish[14] (1962) stipulate incubation of the EC medium in a water bath at 44.5°C ± 0.2°C or in an air, water-jacketed incubator at 45.5°C ± 0.2°C. Preliminary studies by Slanetz et. al.[13] which included both of the above techniques indicated that higher fecal coliform densities were obtained when the EC-MPN or MF cultures were incubated at 44.5°C (water bath) than at 45.5°C. (air incubator). However, other investigators are of the belief that there is no significant difference in fecal coliform recovery by the two temperature-incubation criteria. Consequently, the next edition of Standard Methods For The Examination of Water and Wastewater (13th Edition) will specify use of the water bath at 44.5°C ± 0.2°C for fecal coliform assays when the elevated temperature test is used.[24] The new edition of Recommended Procedures for

the Bacteriological Examination of Sea Water and Shellfish will specify choice of a circulating water bath at 44.5°C ± 0.2°C for sea water and shellfish fecal coliform determinations.[25]

With increasing use of the membrane filter (MF) procedure for water pollution studies, application was made for fecal coliform assays.[15] The elevated temperature reaction is used in this procedure for selectivity. With MFC Broth Base as the medium, fecal coliforms appear as blue colonies while non-fecal coliforms appear as green, pink, and white colonies. As with the EC-MPN elevated temperature test, the question of temperature and type of incubator reappears. Geldreich et. al.[15] received good correlation between EC-MPN and this MF procedure (44.5°C ± 0.5°C - water bath) on fresh water samples and sewage influent. Slanetz[13] reported poor correlation with MF procedures at 44.5°C ± 0.5°C (water bath) on sea water samples. However, with the procedure of Geldreich et. al. - but using an air, water-jacketed incubator at 45.5°C, he reported that it was a direct, efficient method for estimating fecal coliform densities in sea water samples. A report by the Northwest Shellfish Sanitation Laboratory disclosed that the procedure of Geldreich et. al. was encouraging for the enumeration of fecal coliforms in sea water when a water bath and incubation temperature of 44.5°C ± 0.2°C was used. The range of the MF technic when compared to the EC-MPN method was 0.13 to 4.40 with a 50 percentile level of 0.74. The median for 46 samples was 0.75. Fecal coliforms were confirmed in 88% from typical blue colonies.

Green colonies, although few in number, had a higher percentage of confirmation (89.5%). More than 25% of the white colonies were confirmed as fecal coliform colonies. With fresh water samples, for blue, green and white colonies, confirmation percentages were 96.7, 8.90 and 20.0 respectively. Work done on estuarine samples at the North Atlantic Water Quality Management Center Laboratory, Edison, N. J. showed that 32 of 45 samples gave MF fecal coliform values that fell within the 95% confidence limits of the EC-MPN test.[27] Problems were encountered in differentiating green colonies from the typical fecal coliform blue colony. Since a high percentage of these green colonies confirm as fecal coliforms, it was suggested that perhaps these colonies should be included in the typical colony classification.

In summary, the elevated temperature test for separating fecal and non-fecal coliform bacteria gave results comparable to all other methods. The MF application provided simplicity in technique and results at the end of 24 hr. An ideal test for separation was, however, not achieved primarily because of the inability to obtain coliform bacteria from known sources for evaluation of the test procedure as to its specificity, sensitivity and stability of biochemical reactions. In salt water, some differences were observed between the MF and comparative EC-MPN procedures. Not enough comparative data has been accumulated to specify the exact relationship. There is some question on fecal coliform colony identification with the MF procedure. Characteristic fecal coliform colonies on the MFC - broth base are blue colored colonies, however, some green and white colored colonies

have been confirming as fecal coliforms. As a result, the Northwest Shellfish Sanitation Laboratory report[26] concluded the following:

- If fecal coliform organisms are to be considered in the bacteriological examination of shellfish growing areas - then efforts should be made to make further evaluations of this and other MF techniques for the enumeration of this group.

- The main deterrent to the use of MF techniques in shellfish growing areas is the lack of sensitivity. From available data, many coliform colonies were grown on the filter but could not be enumerated due to a lack of typical color formation.

The present philosophy is directed toward a greater use of the fecal coliform bacteria as determined by the elevated temperature test using MF and MPN techniques. For example, the National Technical Advisory Committee To The FWPCA On Water Quality Criteria[16] suggested that total coliform criteria are not satisfactory as indicators of the presence of pathogens in recreational waters. In water, total coliforms that may be of fecal origin may range from less than 1% to more than 90%. Considering the 1% level, a limit of 1000/100 ml total coliforms would impose a non-justifiable limitation on the availability of water for contact recreation. However, at the extreme level (90%) the limit of 1000/100 ml would constitute a threat to the health of individuals in contact recreations. As a result, a recommended standard was proposed by the committee for contact recreation waters. These waters shall not exceed a geometric mean of 200 fecal coliforms

per 100 ml, nor shall more than 10% of the total samples during any 30 day period exceed 400/100 ml. For recreational waters (other than contact sports) fecal coliform counts shall not exceed a geometric mean of 1000/100 ml, nor equal or exceed 2000/100 ml in more than 10% of the samples. The standards on contact recreational waters were based on studies conducted at the Great Lakes (Michigan) and the Inland River (Ohio). Epidemiological data showed the detection of health effects at coliform levels of 2300 - 2400/100 ml. Ohio River data showed that the fecal coliforms represented 18% of the total coliforms. As a result, it would be expected that detectable health effects may occur at a fecal coliform level of about 400/100 ml.

Recent studies have provided some information on the indicator-pathogen relationship. Raritan Bay data revealed salmonella at a coliform density of 54/100 ml (MF) and at a coliform MPN of 230/100 ml. The hard clam, Mercenaria mercenaria contained salmonella when the MPN coliform count was 20/100 g. Salmonella was isolated from the overlying water of shellfish beds which contained an MF coliform count of 88/100 ml.[17] In another report,[18] data gathered in stream surveys showed little apparent correlation between levels of total or fecal coliforms and the isolation of salmonella. These enteric pathogens were isolated at total coliform densities of less than 1000/100 ml and fecal coliform densities of less than 150/100 ml. In a stream several miles below the outfall which discharged unchlorinated domestic waste, salmonellae were isolated even though a 90% reduction of

fecal coliforms occurred. It was concluded by the authors that there is a high probability of isolating salmonella when high fecal coliform densities exist. Data have shown that it is also probable to detect salmonellae at low coliform densities especially if inadequately disinfected waste sources are present. Depending upon existing environmental conditions, slight reductions in salmonellae may occur, while fecal coliform reductions are of a large magnitude. As a result, low fecal coliform values, of themselves, are not sufficient to indicate freedom from these pathogenic forms.[18]

The total acceptance of the results and interpretation of this latter study (18) must be critically evaluated because of the following reasons.

1) The technique used to isolate salmonella is not quantitative. Gauze pads immersed in the water for initial isolation are exposed to the sample water for periods up to 3 days. Therefore, the volume of water passing through the pad is not known. In addition, enrichment techniques used allow for multiplication of the organisms so that detectable numbers are reached. For fecal coliform assays, known volumes of sample are used and quantitation is achieved by the use of selective media and direct counting as with the MF technique or a most probable number derived from MPN statistical probability tables. One must realize the inherent fallacy in trying to compare the 2 types of results with respect to quantitative interpretation.

2) The sensitivity of the salmonella procedure for water analysis has not been fully established. As a result, a negative isolation result

does not ensure the freedom from salmonella. It only means that the pathogens were not detected by the method used.

3) There are over 1000 known salmonella serotypes and all of these do not react the same to the bacteriological testing system used. Therefore, the report cited above (18) which includes only one primary isolation plating medium becomes specific for those serotypes capable of growing under those conditions. Consequently, the use of such a system has been challenged.

4) Survival rates of Salmonella typhimurium as ascertained in that same report were carried out under laboratory conditions using specific wastes which do not reflect the natural stream waste components and concentration. Under these conditions it was noted that S. typhimurium persisted for longer periods and in higher numbers than did the fecal coliforms. On the other hand, Geldreich et. al.[19] found that in storm-water run-off the die-away pattern of S. typhimurium resembled that of the fecal coliforms - with the fecal coliforms persisting in higher numbers than S. typhimurium after 14 days. Slanetz and Bartley[20] reported that the survival curves of salmonella and E. coli were similar, however, the salmonella die-off rate was somewhat slower. If the fecal coliform bacteria are to be used as water quality indicators and standards developed according to the presence of these pathogens - then the above discrepancies must be reconciled.

Another approach used to establish fecal coliform standards is the derivation of densities based on the relationship of coliform to fecal coliform bacteria. A present bacteriological standard for the certification

of waters for the harvesting of shellfish for interstate transport is a coliform median MPN not exceeding 70/100 ml and not more than 10% of the samples ordinarily exceed an MPN of 330/100 ml when a 3-tube decimal dilution test is used. The fecal coliform group has been recommended as a substitute standard for cer·.fication of these waters.[21] A fecal coliform median MPN of 7.8/100 ml was suggested as being equal in significance to a median coliform MPN of 70/100 ml. The number was derived from a statistical analysis of data collected from 6 states and 3 Canadian Maritime Provinces. Objections were raised in the use of such a specific coliform - fecal coliform relationship as ascertained by statistical analyses over a wide range of conditions which may or may not be applicable to specific areas which differ and are subjected to varying degrees of bacteriological contamination from a variety of sources.[22] When the relationship between fecal coliforms and coliforms were studied for several tidal areas on Long Island, N. Y.[22] data demonstrated that this relationship varied with the source of pollution, level of treatment provided for the incoming waste, character of receiving water and the amount of precipitation on the watershed. The proposed fecal coliform standard did not compare with the values received for fecal coliforms found in waters with a median MPN of 70/100 ml. Equivalent values for a median coliform MPN of 70/100 ml ranged from 15 to 67/100 ml. The author concluded - that the setting of standards should be based on the level of public health hazard associated with use of the water.

In the above approach, there is the attempt to develop complimentary

standards utilizing the present coliform standards which were based on studies conducted in 1941.[23] Simply, the standard of 70 coliform bacteria/100 ml was based on the ratio of E. typhosa to coliforms and the factors which affected the ratio. In theory then, some believe that the derivation of such a complimentary standard would inherently reflect the pathogen (salmonella) relationship to fecal coliforms. In practice this may not be so since both fecal coliform and salmonella classification and technology have changed since that time.

Ohio River data[16] showed that the fecal coliforms represented 18% of the total coliforms. Data from tidal estuaries which receive a continuous input of raw and/or inadequately treated sewage gave high percentages of fecal coliforms (North Atlantic Water Quality Management Center, FWPCA, Edison, N. J.) Of 1033 samples studied from New York Beach areas, the fecal coliforms represented 47.1% of the total coliforms. Of 471 samples from Jamaica Bay, fecal coliforms represented 30.1% of the total coliforms and of 2500 samples from Raritan Bay, fecal coliforms represented 42.7% of the total coliforms. It is apparent that the percentage of fecal coliforms will vary from one location to another. Distance from the sewage outfall, salinity, heavy metals associated with seawater, precipitation and surface runoff will affect fecal coliform percentages.

Application of MF Technique for Sewage Analysis

At the present time the twelfth edition of Standard Methods For the Examination of Water And Wastewater includes two methods for the assay of coliform bacteria in water and sewage. These are the Most Probable Number Test (MPN) and the membrane filter technique (MF). The more rapid MF procedure can be used provided that parallel testing has shown that the MF results are equivalent to that given by the MPN procedure. When compatible, the less involved and more rapid procedure has gained wide acceptance and usage in bacterial assays for water and sewage.

In 1957, McKee et. al.[28] published a report on the effects of physical and chemical disinfection of sewage when a membrane filter technique was used to assay coliform bacteria. Three methods of physical disinfection were investigated - heating, ultrasonic vibration and high-speed mixing. The membrane filter results were generally comparable in magnitude with

the MPN values. However, when chlorine was used as the disinfecting agent, great disparity and lack of agreement between the MPN and MF values was observed. Without exception, the membrane filter results were lower than the MPN data. These results were based on domestic sewage collected from sewer trunk lines, returned to the laboratory, allowed to settle and then disinfected with various amounts of chlorine. In an attempt to explain this disparity, two hypotheses were proposed by the authors of this report. The first, points out the clogging of the membrane pores and the formation of an organic mat which prevents bacterial access to the nutrient that diffuses through the membrane filter. Or - despite the dechlorinating procedure used, the mat may contain chloro-organic substances which are toxic to coliforms in close proximity. The presence of a mat on the membrane is most obvious, especially when the residual coliform density is less than 50/100 ml thus necessitating the filtration of at least 10 ml of sewage to obtain a statistically significant number of coliforms on the membrane.

The second hypothesis suggests that hypochlorous acid (HOCl) kills bacteria by cell penetration and oxidation of critical enzyme systems irreversibly. However, in most chlorinated sewages, monochloramine is the predominant disinfectant agent and compared to HOCl it is a weaker oxidant. As a result, the reaction with NH_2Cl can be reversible. When such inactivated cells are present on the membrane and have limited access to nutrient material, growth does not occur. However, with the MPN procedure where lactose or lauryl tryptose broth is used, these inactivated

cells are in a medium rich in organic matter. The NH_2Cl molecule that originally diffused into the cell - when such molecules are numerous, may now diffuse outward from the cell into solution. The cell is now able to grow and carry out normal metabolic processes.

Unfortunately, the above report has gone unnoticed and substantiating reports in the literature have not been forthcoming. At the present time a number of agencies utilize the MF procedure to evaluate the performance of sewage treatment plants in removing or destroying bacteria. If the above report is valid, then the MF procedure for assaying coliform bacteria in chlorinated effluents should be abandoned or modified in such a way that reliable results may be obtained. Such a decision would have far reaching implications and question previous sewage treatment plant data generated by this method. Because of the urgent need to substantiate the above report and if possible to detect the operating mechanism and correct this discrepancy, a preliminary investigative program was initiated.

Laboratory equipment was transported to a primary sewage treatment plant and analyses were performed at the site of sample collection. Sewage effluent was analyzed before and after chlorination by MF and MPN procedures. Results of MF coliform analyses on non-chlorinated effluent are comparable in magnitude with the MPN values. However, 70% of the MF values on chlorinated effluent failed to fall within the 95% confidence limits of the MPN test.

An attempt to increase the availability of nutrient medium to the organisms deposited on the membrane was carried out by using an agar base

M-Endo-MF medium. (In the conventional method, this same medium without agar is used to saturate the pads on which the membranes are placed.) Parallel samples, (pad and agar) gave similar MF values. The disparity between the MF and MPN values still existed even though more moisture and nutrient saturation of the membrane occurs with the agar medium. It was, however, observed that coliform colonies on the agar base medium were larger and displayed a more brilliant green, metallic sheen on the surface of the colonies.

Pre-enrichment techniques were then considered in an effort to increase coliform recovery on the membrane. Duplicate sets of membranes were prepared by filtering several volumes of chlorinated effluent. One set was placed on M-Endo-MF agar and immediately incubated. The other set of membranes were placed on pads saturated with lauryl tryptose broth (LTB) for 2 hours. After this time period the membranes were transferred to M-Endo-MF agar plates and incubated. Concurrent MPN tests were performed on the same samples. Pre-enrichment with LTB for 2 hours gave no increased recovery over the non-enriched membrane filters. MPN values were still significantly higher than the MF enrichment values.

Blending of the effluent appears to somewhat reduce the organic mat on the membrane. Chlorinated effluent samples blended for 2 minutes at high speed gave dramatically increased numbers of coliforms in both MF and MPN procedures. In every case, the non-blended effluents exhibited lower

numbers of coliform bacteria on both test procedures. With the blended samples, MF values began to approach those obtained by the MPN procedure. Additional data is needed to describe the precise effects and to see whether the MF values will continue to approach those obtained by the MPN procedure. Blending of the non-chlorinated effluents did not show significant coliform increases over the non-blended sample by both MF and MPN procedures. This type of comparative data, (blending and no blending of chlorinated and non-chlorinated effluent) hopefully, will give some insight into the question whether or not blending is effecting higher counts as the result of simple particle destruction; whether the chloro-organic clumps are now more affected by the dechlorinating agent or whether it is a combination of both. Similar studies on secondary sewage treatment plant chlorinated effluents will be required in order to see if the phenomenon is present with such treatment practices.

The data from the blending experiments suggest that clumping and chloro-organic aggregates may be a more realistic explanation as to why coliform counts are lower by the MF technique. This data can also explain - in part - for the occurrence of so called coliform "after growth" that has been observed in receiving waters.

One can extrapolate from the above study and surmise that the MF fecal coliform test will encounter the same problem. As a result, future studies will have to investigate this effect on the MF fecal coliform procedure.

Conclusions

A critical review of the methods of detection, enumeration and interpretation of the coliform group of bacteria has developed the following points.

1. The fecal coliform bacteria are a more realistic microbial indicator of hazardous pollution. As a result, attempts are being made to establish recreational water and shellfish harvesting water standards based on fecal coliform densities.

2. The (EC) elevated temperature test for enumerating fecal coliform bacteria is not a perfect test, however, for separating fecal and non-fecal coliform bacteria the test provides reliable results and is superior to other procedures. The MF application (by Geldreich) provides simplicity in technique and reliable results at the end of 24 hr. when fresh water samples are analyzed.

3. The EC-MPN fecal coliform procedure is a reliable procedure for estimating fecal coliform densities in fresh water, sewage and salt water. The incubation conditions have been established at $44.5^{\circ} \pm 0.2^{\circ}C$ using a water bath so designed to provide uniform temperature distribution throughout the bath. The *E. coli* - MPN procedure differs from the EC-MPN procedure in that it tends to only measure the classical *E. coli* IMViC types. As a result, it is too restrictive and excludes other fecal coliforms which have been demonstrated in the feces of warm blooded animals. Such a narrow definition of the fecal coliform group involves the risk of not detecting

hazardous conditions, especially at minimal levels of contamination. For FWPCA surveillance and pollution control programs such a system is not adequate in assessing the bacteriological quality of the water.

4. The relationship of the fecal coliform bacteria to the presence and numbers of enteric pathogenic bacteria in water has not been fully established. Reports on the subject are conflicting and this is due to the lack of a reliable, sensitive and quantitative procedure for enteric pathogen determination. In order for meaningful and realistic fecal coliform standards to be developed - such a relationship must be established. Fecal coliform standards based on a relationship to total coliform bacteria do not appear to have merit since such a relationship is affected by source of pollution, level of treatment provided for the incoming waste, character of the receiving water and the amount of precipitation on the watershed.

5. Preliminary studies indicate that fecal coliform survival patterns are similar to that of the salmonellae. Conflicting reports arise when different experimental designs are used to develop survival patterns. Tests conducted under laboratory conditions do not take into account natural factors encountered in the environment. As a result, up-to-date information on <u>in situ</u> survival is lacking.

6. The membrane filter technique (MF) using MFC broth base and incubation in a water bath at 44.5°C correlates well with the EC-MPN technique when fresh water samples are analyzed. Problems were encountered when sea water samples were analyzed by this method. A large percentage of colonies designated as non-fecal according to definition of the test proved to be fecal coliforms when confirmation tests were performed. It is not known

whether the two hues are so similar and therefore difficult to separate or whether the greenish-type colony is a distinct type and should also be designated as a fecal coliform colony. Additional data on the correlation of this MF procedure with the EC-MPN technique in sea water samples are needed.

7. Preliminary data show that the MF coliform procedure produces results which are significantly lower than values received by the MPN procedure when chlorinated sewage effluent is analyzed. Further study is required to determine the mechanism (s) involved and to modify the MF technique accordingly so that reliable data can be obtained.

Immediate Research Needs

1. Define the effect of salt water on the specificity of the MF fecal coliform procedure and modify accordingly so that acceptable recovery and colony differentiation can be achieved.

2. Determine the precise effects of chlorine used in sewage disinfection on the performance of the coliform membrane filter procedure. Adjust and/or modify present MF system to remove detrimental effects of chlorine on membrane - biological system.

3. Determine *in situ* survival patterns of fecal coliforms and pathogenic bacteria in fresh and salt water environment. Initial studies with pathogens to include *Salmonella* serotypes.

4. Development of a reasonably rapid system for the detection and quantitation of *Salmonella* in aqueous systems.

5. Establish the relationship of the fecal coliform bacteria to the numbers of enteric pathogenic bacteria in fresh and salt water.

6. Development of a delayed fecal coliform membrane filter procedure.

Research Grant Programs

Several grant programs are in effect which may fulfill some of the above immediate needs.

- Item 5 above:

Research grant number WP 123 - "Indicator-Pathogen Density Relationships in Tributary Streams". - University of Detroit. 1967-1970.

This study involves a bacteriological examination of the waters of a small surface tributary which is typical of many streams draining suburban areas. The object of the examination is to characterize the quantitative relationships existing between densities of fecal pollution indicators and of *Salmonella* species existing under all nutrient and environmental conditions which will pertain along the course of the representative stream. (This study does not include salt water environments.)

- Item 6 on previous page:

A grant program involving the development of a delayed fecal coliform procedure is in progress at the University of Cinn. Similar research, however, on an independent basis is being considered at the Water Research Laboratory, Water Supply and Sea Resources Program, USPHS and by the research and development unit of the Millipore Corporation in Bedford, Mass.

To my knowledge, there are no concentrated programs involving the needs specified in items 1, 2, 3 and 4 on the preceding page.

Planned Accomplishments
FY 69

1. Determine the precise effects of chlorine on coliform membrane filter procedure when effluents of primary and secondary treatment plants are analyzed.

2. Determine mechanism involved in low recovery of coliform organisms from chlorinated sewage treatment plant effluents on membrane filter systems.

3. Determine the feasibility of pre-enrichment techniques including amino acids for minimizing detrimental effect of chlorine and increasing coliform recovery on membrane filter systems.

4. Define the effect of salt water systems on the specificity of the MF fecal coliform procedure.

5. Optimize color indicator system of MF fecal coliform procedure to include maximum recovery of organisms from salt water systems.

Planned Accomplishments
FY 70

1. Continue and complete studies on chemical indicator system of MF fecal coliform procedure.

2. Develop membrane filter medium for the recovery of Salmonella from aqueous systems.

3. Ascertain recovery efficiency using known salmonella serotypes inoculated into fresh and salt water samples containing normal mixed microbial flora.

4. Determine the specificity of the medium by studying ability or negative growth response of Gram Negative organisms commonly found in fresh and salt water.

Planned Accomplishments
FY 71

1. Prepare salmonella serotype antigens for the production of poly and specific antisera.

2. Determine antisera titer and cross-reactivity by agglutination and precipitation tests.

3. Develop and refine serological techniques and reagents for use in the fluorescent-antibody-membrane filter method for detection and identification of salmonella serotypes.

4. Conjugate antisera of adequate titer with fluorescein isothiocyanate dye and test reagents by using FA slide, direct agglutination method.

5. Determine the most suitable filter (based on least amount of autofluorescence) and optical combinations for use in the membrane procedure.

Planned Accomplishments
FY 72

1. Determine the performance of the FA-membrane procedure in detecting and identifying salmonellae in polluted fresh and salt water. Concomitant confirmation by conventional biochemical and serological procedures are to be performed.

2. Remove additional non-specific staining and cross-reactivity as found in above evaluation. To be accomplished by removing non-specific staining characteristics of the serum and by absorbing the cross-reactivity components of the serum.

3. Complete evaluation (as in 1 above).

Planned Accomplishments
FY 73

1. Develop survival chambers utilizing principles of dialysis for *in situ* determination of survival patterns of fecal coliforms and pathogenic bacteria. Such data to be compared to results of survival studies utilizing continuous culture techniques.

2. Isolate fresh cultures of fecal coliforms and salmonella serotypes from polluted sources. Organisms are to be used as test organisms in survival studies.

3. Immerse into fresh and salt water - dialysis systems containing known numbers of pathogens and indicator bacteria in saline; suspended in sewage effluent; and suspended in buffered water. Extract aliquots of the test sample at specific periods of time and determine death curves of the test organisms.

4. Survival rates will be determined in clean, slightly polluted and heavily polluted fresh and salt water during the four seasons of the year. For survival determinations in the marine environment, waters with chloride ranges from 5000 to 18,000 mg/l will be selected.

5. Using continuous culture techniques, study growth characteristics of fecal coliforms and pathogenic bacteria under varying conditions of temperature and salinity.

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Fecal Streptococci

Introduction

The fecal streptococci were proposed as indicators of fecal contamination as early as 1910. However, in the United States application of this group of bacteria to pollution detection did not take place until the post World War II period. At the present time, the test for the presence of fecal streptococci is listed as a tentative method in the 12th edition of Standard Methods for the Examination of Water and Wastewater. Its tentative status indicates that full acceptance of the test procedure is lacking and that certain inadequacies prevail. Despite this condition, many investigators feel that fecal streptococcus data may supplement information provided by the coliform group of organisms. This was especially true in the past when it was difficult to establish the fecal origin of the coliform organisms. However, with the advent of the EC elevated temperature test for the separation of fecal and non-fecal coliforms, the initial need to further characterize the pollution source was minimized. Universal usage was further hindered by the lack of suitable recovery media, lack of agreement on the significance of specific species or sub-groups, host specificity, terminology and classification of the fecal streptococci. The lack of a rigid definition and use of a number of terms to designate the bacteria in question led to added confusion. The terms streptococci, enterococci, group D streptococci and fecal streptococci have all been used to

denote the bacteria in question. One can, therefore, sense an evolution of changing philosophy, definition and methodology in the quest for acceptance as a microbial indicator of pollution.

Definition

Many bacteriologists, especially of the French school, have long recognized the occurrence in the human intestine of a characteristic streptococcus, which usually occurs in pairs of ovoid cocci and short chains. Thiercelin[1] in 1890 described the characteristic morphology and called it the Enterococcus. The organism exhibited marked resistance to heat[2,3,4,5] and specific biochemical properties. Additional fermentative properties were revealed, and keeping in mind the origin of the bacterium, Andrewes and Horder[6] assigned the name Streptococcus faecalis. Thus — the beginning of the streptococcal indicator and subsequent development of other group organisms.

Members of the streptococcal group of bacteria have widely different environmental influences in the life of man and other animals. Species range from those that are capable of producing disease in susceptible man and animals with different degrees of virulence. This may be carried out through the intermediate types of parasitic nature with mildly hazardous to non-hazardous relationships to animal life and to the saprophytic species used in the processing or flavoring of food products for human consumption. Consequently, such a broad spectrum of environmental activities in a single genus

poses difficult problems in the choice of a practical system of classification. Numerous divisions in groups or species, involving biochemical reactions or serological analysis and/or other characteristics have been proposed by investigators. To review all of the taxonomic classifications of the genus Streptococcus is beyond the scope and objectives of this discussion on the application of a small portion of this group to the detection of fecal pollution in water. A certain amount of classification, however, must be presented to accurately describe and identify the species that are most useful in pollution detection.

The genus Streptococcus has the following common characteristics which distinguish it from other genera of bacteria. These include:

1. Gram positive, ovoid cells with a diameter of 0.5 to 1.0 microns and occurring in chains of 2 or more cells. They more commonly occur in the diplococcus state;
2. The cells may exhibit pleomorphism under certain environmental conditions;
3. The chief metabolic product from the fermentation of carbohydrates is d-lactic acid;
4. Organic acids or volatile compounds are not produced or only in trace amounts;
5. Do not produce carbon dioxide or in very small quantities from sugar fermentation;

6. Are insoluble in bile;

7. Are aerobic and facultatively anaerobic microorganisms;

8. Are negative for catalase production and contain no cytochrome systems.

Some exceptions to the above have been reported in the literature, however, these are academic in nature insofar as the use of the streptococcal group as indicators of pollution.

As was noted previously, the streptococci are among the most difficult groups of bacteria to classify. One of the earliest classifications was proposed by Brown (1919)[7] who divided the organisms into three groups according to their activity on blood agar:

1. Alpha streptococci, which produced a greenish coloration (methemoglobin formation) of the medium and partial hemolysis in the immediate vicinity of the colony.

2. Beta streptococci, which produced clear, colorless zones (complete hemolysis) around the colonies.

3. Gamma streptococci, which showed no effect on blood agar.

Since activity on blood agar could be related to certain pathogenic forms of streptococci, its importance was medically oriented. This type classification, however, has no value in pollution investigation.

Serological tests, primarily the Lancefield precipitin technique, have been very useful in the classification of streptococci. Lancefield proposed this serological classification system in 1933.[8] In general, all streptococci,

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except those in the viridans group possess a serological group specific "C" substance, polysaccharide in nature and located on or in the cell wall. As a result, streptococci are placed into serological groups according to polysaccharide antigens as determined by the precipitin technique. One serological group therefore, may comprise more than one species. Altogether, 13 groups have now been recognized, each with its peculiar polysaccharide antigen. These include groups A,B,C,D,E,F,G,H, K.L.M, N and O.

In addition to the group specific polysaccharide antigens, type-specific substances (protein or other polysaccharides) in the form of capsular type-specific antigens detected by the agglutinin or precipitin technique are used in serological classification. Therefore, one Lancefield serological group may possess a number of established serological types based upon the presence of capsular-type-specific antigens. This type of characterization has been useful for the pathogenic streptococci. Work done on pathogenic organisms from human sources and from mastitis in cows, show that groups of organisms from different sources tend to be characterized by specific polysaccharide antigens and within these groups strains may be allocated to a number of types by virtue of a series of other antigens which may be either protein or polysaccharide in nature. The association between the type of polysaccharide and the source of the strains is close, but not absolute.

With the group D streptococci some attempt has been made to distinguish antigenic types within the group. Lancefield[9] defined 3 types while Grumbach and Schnetz[10] noted 7 types. Within the group, Skadhauge[11] distinguished thermostabile somatic O antigens, capsular A antigens and thermolabile K antigens. In cross-agglutination studies it was noted that there was an affinity between the O antigens of Streptococcus faecalis and its varieties, but not between these and members of the tellurite-sensitive group. By cross-absorption, 6 types were recognized in the S. faecalis group. Sharpe and Shattock[12] examined 353 strains of group D streptococci and were able to distribute 281 of them into 24 types by agglutination and precipitin tests. S. faecium was numbered type 25. Sharpe and Fewins[13] found 14 new types of group D streptococci isolated from canned hams and pigs' intestines and numbered them types 26-39.

Serology may therefore, provide a more accurate and rapid means for distinguishing enterococci. For application to pollution studies, sufficient data on the correlation of antigenic types and specific host sources are not available. Also, little relationship has been noted between serological type specificity and species specificity as determined by their respective physiological characteristics. Several cases have been cited where certain strains possess common type antigens but belong to different serological groups. As a result, serology has not been extensively used with the fecal streptococci for pollution work.

Sherman[14] divided the streptococci into 4 basic groups: Pyogenic, Viridans, Lactic and Enterococcus. The division represents the most practical application to pollution investigation. Such a division has historical acceptance and encompasses sound bacteriological procedures. It is primarily based on the natural divisions in the group as separated by the maximum and minimum temperatures for growth, plus other supplemental but common physiological characteristics. In such a scheme, however, there is always the difficulty in placing new species, especially those that may contain characteristics of more than one group. As this occurs, the individuality of each group becomes less distinct. For example, S. uberis (Diernhofer)[15], a member of the viridans group possess certain characteristics which would appear to place it in the enterococcus group. S. acidominimus (Ayers and Mudge) also a member of the viridans group could be considered for inclusion into the pyogenic group.

The Four Primary Groups Of The Streptococci:
(According to Sherman)

Group	Pyogenic	Viridans	Lactic	Enterococcus
Growth @ 10°C	-	-	+	+
45°C	-	+(-)	-	+
6.5% NaCl	-	-	-	+
pH 9.6	-	-	-	+
0.1% methylene blue in milk	-	-	+	+
Reductase	-	-	+	+(-)
TST (30 min. @ 60°C)	-	+	+(-)	+

Legend : - Negative test ( ) Rarely
+ Positive test

The interpretation of the above groups as it applies to the determination of fecal pollution in water is:

Pyogenic Group

This group includes most of the pathogenic streptococci. Species belonging to this group are: S. pyogenes, S. dysgalactiae, S. zooepidermicus, S. equisimilis, S. equi, S. sanguis, S. anginosus and S. agalactiae. Members of this group are of essentially no use in the evaluation of pollution in water.

Viridans Group

This group contains S. equinus, S. bovis, S. salivarius and S. thermophilis. Some have considered each of these species as sub-groups rather than individual species. S. thermophilus has no known sanitary significance in the investigation of fecal pollution. S. salivarius group of organisms occupies a controversial position as fecal streptococci and the remaining species or groups are known inhabitants of the alimentary tract of warm-blooded animals.

Lactic Group

The group includes the species S. lactis and S. cremoris. These organisms are not natural inhabitants of the alimentary tract of warm-blooded animals. As a result, they have no sanitary significance in the study of fecal pollution. Their importance lie in other areas of interest. For example, as nuisance organisms they interfere with the detection of the fecal streptococci and their presence may result in erroneous interpretation as

indicating fecal pollution. They have some value as an indicator microorganism for non-hazardous pollution such as wastes from food processing plants.

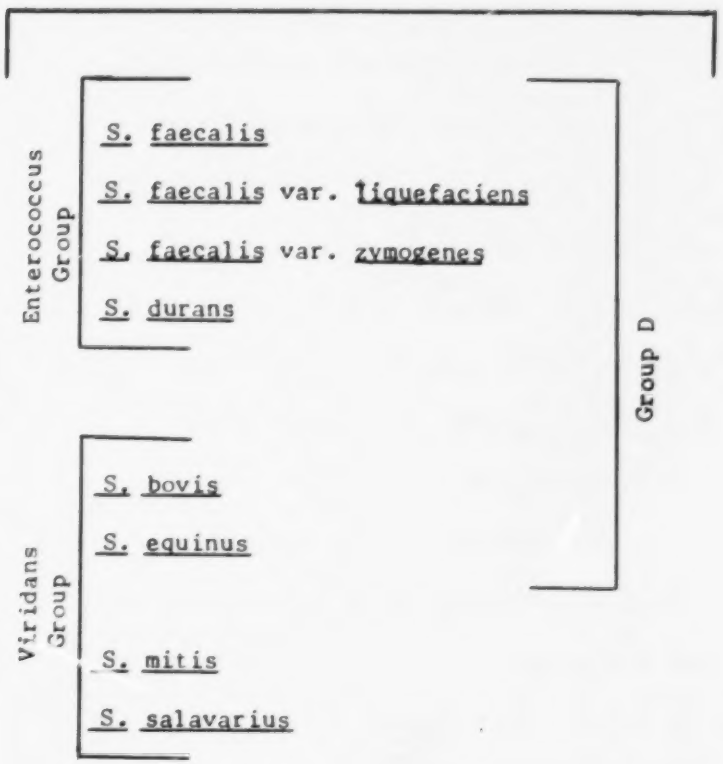
Enterococcus Group

The enterococcus group consists of S. faecalis, S. faecalis var. zymogenes, S. faecalis var. liquefaciens and S. durans. The above species are known inhabitants of the alimentary tract of warm-blooded animals. These are sometimes referred to as the "common fecal streptococci".

The enterococcus group as defined above, follows the strict interpretation of the Sherman classification. This group is considered to be characteristic of fecal pollution and as such are a portion of the "fecal streptococcus group". Several interpretations of the enterococcus group found in the literature have resulted in confusion with this term being meaningless unless defined by the authors. For example, the term enterococci has been used to only indicate streptococcal species belonging to Sherman's Enterococcus Group, while others have included all fecal strains such as, S. mitis, S. salivarius, S. bovis and S. equinus. In Standard Methods,[16] the terms fecal streptococcus and Lancefield group D streptococci are considered synonymous and denote the following species: S. faecalis, S. facalis var. liquefaciens, S. faecalis var. zymogenes, S. durans, S. bovis and S. equinus.

The enterococcus group of organisms, based on a serological classification fall into the original Lancefield group D streptococci. Therefore, the terms enterococcus group and group D streptococci denoted the same organisms. However, there has been an attempt to place additional streptococci, that are normally found in animal feces, in the group D classification. For example, S. bovis strains (viridans group) cross-react with group D sera and cellular extracts made by special preparation exhibit the group D antigen. Because S. bovis is serologically heterogeneous, where many other serological types have been demonstrated, it had not been initially placed in any specific Lancefield group. S. equinus (viridans group) in initial studies did not exhibit any group-specific antigen, therefore, it was not included in any specific Lancefield group. However, physiologically it resembles S. bovis and recently Smith and Shattock[17] demonstrated that the group D antigen can be extracted from disintegrated but not from whole cells. As a result, the organism could be placed into the group D haemolytic streptococci. As a result, based on the demonstration of the group D antigen, the expanded group D would include: S. faecalis, S. faecalis var. liquefaciens, S. faecalis var. zymogenes, S. durans, S. bovis and S. equinus. If the expanded group is accepted, then the terms enterococcus group and Lancefield group D will not denote the same organisms.

Fecal Streptococci



In the above categorization, the common bond is found in the association of all the above organisms with the feces of warm-blooded animals. As such, all are included in the general term, fecal streptococci.

Kenner et. al.[18] studied the distribution of fecal streptococci and biochemical types present in human and animal feces.

Biochemical Reactions of 1092 Cultures
Isolated from Fresh Feces

Biochemical Reaction	Entero-coccus Group	S. mitis-salivarius Group	S. bovis Group	S.equinus-acidominimus Group	Enterococcus Biotype Group
Growth at 45°C	+	(±)	+	+	+
Growth in 6.5 percent sodium chloride	+	-	-	-	∓
Growth at pH 9.6	+	-	-	-	±
Growth in 40 percent bile	+	-	+	±	+
Litmus Milk:					
Acid	+	+	+	-	+
Coagulation	+	+	+		+
Reduction	(+)	Partial	+		+
Peptonization	±				±
Reduction 0.1 percent methylene blue in milk	+	-	-	-	∓
Reduction of potassium tellurite (1:2500)	+	+	- (tr. in 7 days)	(∓)	±
Fermentation sorbitol	±	-	Not used	Not used	±
Final pH in glucose broth	3.9-4.6	4.0-5.8	4.0-4.5	Not used	4.0-5.8
Growth in ethyl violet azide broth:					
Number strains	548	62	37	11	119
Percentage	97.51	80.51	19.89	19.00	57.21
Total number strains isolated	562	77	186	58	208

Seventy-seven strains of the S. salivarius group or 16.3% was isolated from human feces, while none were isolated from the feces of cows, sheep, pig and fowl. S. salivarius occurs commonly in the mouth and can be found in the intestines of humans. The organism resembles S. bovis in many ways, however, it can be readily distinguished by growth at lower temperatures, fermentation of starch and arabinose, its lower tolerance for bile and its absence of precipitation with group D serum. Because it can be found in human feces, the authors considered it to be a fecal streptococcus. Some investigators[19] do not feel that S. salivarius has been established as a normal inhabitant of the intestinal tract and has not been isolated from feces in frequencies that would give it equal status to the other fecal streptococci.

Kenner et. al.[18] also noted the presence of other streptococci in feces which did not fall into any specific biochemical group. The organisms were similar to enterococcus group species but failed in one or two biochemical reactions which are characteristic of the enterococcus group. Such strains were isolated from the feces of humans, cows, sheep, pig and fowl. The density of these organisms was relatively low in human feces but increased progressively in the feces of cows, sheep, pig and fowl. These investigators concluded that such strains could not be properly placed in the enterococcus group since they differ in one or two of the accepted critical reactions

used in the enterococcus group classification. However, due to their close resemblance to the enterococcus group , were tentatively described as enterococcus biotypes. There appears to be some justification for this since they were working directly with fecal samples. Similar biotypes were noted by others [20,21] who found that most of the biotype strains gave positive precipitin reactions with group D sera. As a result, it was stated that because these organisms have been shown to be associated with feces and contain the group D polysaccharide, they can be given fecal indicator status. (More recently —— such organisms were called S. faecalis biotype I, II, III and IV. - Kenner and Haley)

For a practical application to pollution investigation and according to the data developed above, the following categorization can be made:

Fecal Streptococcus Group

1. Enterococcus group (According to Sherman —— including the following species: S. faecalis, S. faecalis var. zymogenes, S. faecalis var. liquefaciens and S. durans.)
2. S. bovis sub-group.
3. S. equinus - acidominimus sub-group.
4. Enterococcus biotype sub-group or S. faecalis biotype group consisting of S. faecalis biotypes I, II, III and IV.
5. S. salivarius sub-group.

By use of standard biochemical tests the above organisms can be identified and separated into groups.

BIOCHEMICAL REACTIONS OF 4,633 CULTURES ISOLATED FROM HUMANS AND OTHER WARM BLOODED ANIMALS*[1]

No. of stains	Growth at 10°C	Growth at 45°C	Growth in 6.5% NaCl	Growth in Broth pH 9.6	Reduction of Methylene blue (0.1%)	Reduction of K_2TeO_3 1:2500	Final pH in 1% glucose	Hydrolysis of starch	Arginine hydrolysis	Litmus Milk: Acid	Litmus Milk: Coag	Litmus Milk: Reduct	Litmus Milk: Pepton	Fermentation 1% Sorbitol	Species
2,706	+	+	+	+	+	+	3.9-4.6	0	+	+	+	(+)	±	+	S. fecalis var.
43	+	+	+	+	+	0	4.0-4.5	0	+	+	+	+	0	0	S. durans (faecium)
823***	(+)	+	+	+	(0)	±	3.9	0	(+)	(+)	(+)	(+)	(0)	(+)	S. fecalis biotype I
	(+)	+	+	0	±	±		0	(+)	(+)	(+)	(+)	(0)	(+)	S. fecalis biotype II
	(+)	+	0	+	±	±		0	(+)	(+)	(+)	(+)	(0)	(+)	S. fecalis biotype III
	+	+	0	0	0	±	6.5	0	(+)	(+)	(+)	(+)	(+)	(+)	S. fecalis biotype IV
698	0	+	0	0	0	0	4.0-4.5	+	0	+	+	(+)	0	not used	S. bovis
93	0	+	0	0	0	(0)	4.0-4.5	+	0	0	0	0	0	not used	S. equinus
78	0	(+)	0	0	0	+	4.0-4.5	0	±	+	+	+	0	not used	S. salivarius
192	(+)	+	(+)	(+)	(+)	+	4.0-4.5	+	+	+	+	(+)	±	not used	Atypical ** S. fecalis var.

*All strains were catalase-negative; all grew in 40% bile broth, except S. salivarius.

**Atypical strains usually isolated from fermenting vegetation, isolated only from cat feces (10 strains), and dog feces (182 strains).

(+) = usually positive; (0) = usually negative; ± = variable.

***Biotypes arbitrarily based on growth in 6.5% NaCl broth and pH 9.6 broth, allowing four types.

[1]B. A. Kenner and P. C. Haley (To be published 1967)

While the above reactions will reduce the streptococci to species, the procedure is highly involved, lengthy and may not be desirable for survey investigations. For sanitary interpretation, simple temperature-growth tests can be used. Geldreich et. al.[22] have used such an abbreviated biochemical scheme. The presence or absence of growth at 10° or 45°C divides the streptococci into 4 different groups:

1. Growth at 10° and 45°C in brain heart infusion broth (BHI) with subsequent positive confirmation in 6.5% NaCl in BHI would be indicative of an enterococcus group organism.
2. Growth at 45°C only (BHI) would indicate organisms of the S. bovis, S. equinus, S. salivarius groups. Separations can be made based on the ability of S. bovis and S. equinus to hydrolyze starch while S. salivarius does not have that ability. S. equinus does not produce acid from lactose, while S. bovis only produces acid from this carbohydrate.
3. Growth at 10°C only (BHI) would indicate the low temperature group which includes the lactic acid group of streptococci commonly found in soil and on plants. These organisms are therefore, of no use in fecal contamination studies and usually do not grow on KF medium.
4. Growth at neither 10° or 45°C (BHI) indicates bacteria belonging to the pyogenic group of streptococci and are rarely found in water. This group has no sanitary significance in water pollution studies.

The above abbreviated scheme has one disadvantage in that the enterococcus biotype group organisms will not be distinguished from the enterococcus group species. On the other hand, if animal pollution predominates, the S. bovis and S. equinus groups will be detected and identified by this scheme.

The biochemical examinations require strict adherence to temperature requirements and accuracy in bacteriological manipulations. Even with good techniques, some of the tests vary in their reliability. For example, the time-temperature test (used in the Sherman classification) for determining the ability of organisms to survive exposure to 60°C for 30 minutes depends upon such factors as the medium in which the cells are grown and the number of cocci present at the start. Different readings may be obtained with the same organisms on different days. Therefore, the results must be interpreted with discretion. In the 45°C test, the temperature of the water bath must be controlled ±0.1°C. In the pH 9.6 test —— difficulty is encountered in maintaining the high pH level. The reaction of the medium therefore, must be checked and adjusted prior to incubation and should be checked again after incubation. Several investigators have advocated incubation in a jar containing soda lime, so that changes in the reaction may be minimized. Growth in the presence of 6.5% NaCl in BHI sometimes is so poor and difficult to read so as to render the test of little value.

Methodologies used in carrying out the biochemical tests have undergone repeated modification while no attempt has been made to standardize and compile uniform sets of procedures. Laboratories therefore, differ in the preparation, application and control of media used in biochemical characterization. There is a good deal to be desired in the biochemical schema and methodology presently used for identification.

Application

The significance of the enterococci lie in the fact that they are present in feces, sewage[23] and polluted waters.[24] The enterococci were not found in pure water and in areas devoid of human and animal life.[25] It was believed that these organisms did not multiply in water and soil.[26,27] Four basic tenets were therefore, established:

(a) the abundance of the enterococci in the feces of man;
(b) their absence in pure water;
(c) their inability to multiply in pure water and
(d) their inability to survive for long periods in pure water.

A fifth premise could be added on information by Medrek and Litsky[28] who showed that virgin soil was free of enterococci. The establishment of the above basic requirements were instrumental in the further development of these organisms as indicators of fecal pollution.

Further consideration in the use of the fecal streptococci as indicators of fecal pollution came when Cooper and Ramadan[29] demonstrated that certain species or groups predominate in human feces while others predominated in animal feces. This finding was verified by other investigators[30] who stated that the presence of typical S. faecalis strains would indicate pollution of human origin while the presence of S. bovis strains indicated animal origin. Data on the distribution of fecal streptococci in the feces of humans and domestic animals, indicated that this situation was not that clear-cut.[18] The presence of S. faecalis alone did not always denote pollution from human sources. For example, enterococcus group organisms could be found in the feces of humans, cows, sheep, pig and fowl. However, according to the authors, sufficient data was available on several groups of fecal streptococci that would indicate an application to source distinction. It was noted that a high percentage of enterococcus group organisms, demonstration of S. salivarius and very few strains of the enterococcus biotypes would indicate pollution of human origin. S. bovis or S. equinus groups would indicate animal sources and as the numbers of these organisms increase one could expect that the quantity of animal fecal material was also increasing. Fecal pollution by fowl would be indicated by a large percentage of enterococcus group and biotype group organisms. In situations where all 5 types (enterococcus group, S. salivarus, S. bovis, S. equinus and enterococcus biotypes

groups) are present, interpretation would be difficult. Physiological characteristics tend also to make interpretation difficult. For example, S. salivarius is a very sensitive and fragile organism, dying very rapidly when exposed to environmental factors. Besides being sensitive to the higher pH levels, S. bovis and S. equinus succumb within 24 hrs. exposure. Water samples taken at points in a stream which exceeded 24 hrs. travel time from the point of discharge would not be expected to yield S. bovis and S. equinus.

That undue stress was being placed on S.faecalis and the other enterococci as indicators of human pollution was further shown by Ostrolenk and Hunter[31] and Mundt.[32] They found that the organisms occur quite commonly in wild animals. Seventy-one percent of various mammalian species showed enterococci; 86% of the reptiles, including lizards, snakes and turtles displayed enterococcus group organisms. S. faecalis var. liquefaciens (88) or 28.6% isolation; S. faecium (87) 28.2% isolation and S. faecalis (37) 12.0% isolation —— were the predominant species.

Generally speaking, the higher one proceeds up the taxonomic scale, the more frequently the bacteria are found. Mieth[33] suggested that the occurrence of enterococci is food-dependent. Isolations of enterococci from herbivores or subsurface ground dwellers were low, while occurrence increased with the larger animals who displayed varied diets.

As a result, it was concluded "that the ubiquity of S. faecalis and S. faecium in man and animals does not seem to warrant one in restricting S. faecalis as the indicator organism of pollution, nor to make a distinction between the human and animals with respect to source."[34]

The presence of enterococci in the feces of man, domestic and wild animals which even include poikilothermic forms has been adequately established. Because of this wide range of resistance and tolerance, it was suspected that the organisms would be found to exist under varied environmental conditions.[35] Mundt found that enterococci may be chance contaminants of plants. Enterococci were recovered from 42% of 116 genera or families of plants in areas remote from man. In agricultural areas, approximately 33% of all flowers yielded enterococci. The enterococci occurred on some species of plants, never on others and sporadically on the remainder. They are rarely found on grasses and cereals, however, they were isolated from corn. On certain types of plants, S. faecalis var. liquefaciens was found to be quite common.[36] In a subsequent study[35] the majority of isolates from plants fermented lactose very slowly and produced a rennin-like curd in milk, followed by stratiform digestion. This was reported as being similar to the dominant strains recovered from animals, but unlike those isolated from humans where the proteolytic strains produced a hard, acid curd. This was, however, of no value in the distinction between human and non-human strains because all caseolytic strains produce rennin, however, differing in the rate of lactose fermentation.

According to Geldreich et. al.[37] the presence of enterococci on vegetation may, at least in part, have been derived from insect contact. Other investigators[38,39,40] previously reported the presence of enterococci in various species of insects. In order to establish the levels and streptococcal types on vegetation and insects, Geldreich et. al.[37] performed concurrent coliform and fecal coliform determinations. Results suggested that typical fecal coliforms, as measured by the fecal coliform test, contributed only a small percentage of the organisms associated with insects and vegetation. Therefore, it was concluded that the contribution from warm-blooded animal pollution was a minor factor in both vegetation and dissemination by insects. However, the higher levels of streptococci found on vegetation were explained as being streptococcal types not of warm-blooded origin. For example, of 646 strains isolated from vegetation, 241 (37.7%) hydrolyzed starch, even though all other biochemical criteria were typical of the enterococcus group as defined by Sherman. On the other hand, studies on fecal streptococci from water, warm-blooded animals, cold-blooded animals, insects and soil have never produced S. faecalis strains displaying this characteristic. Other factors that could have contributed to the high levels of enterococci on plants as reported by several investigators were:

(a) some proliferation of enterococcus organisms may occur with insects;

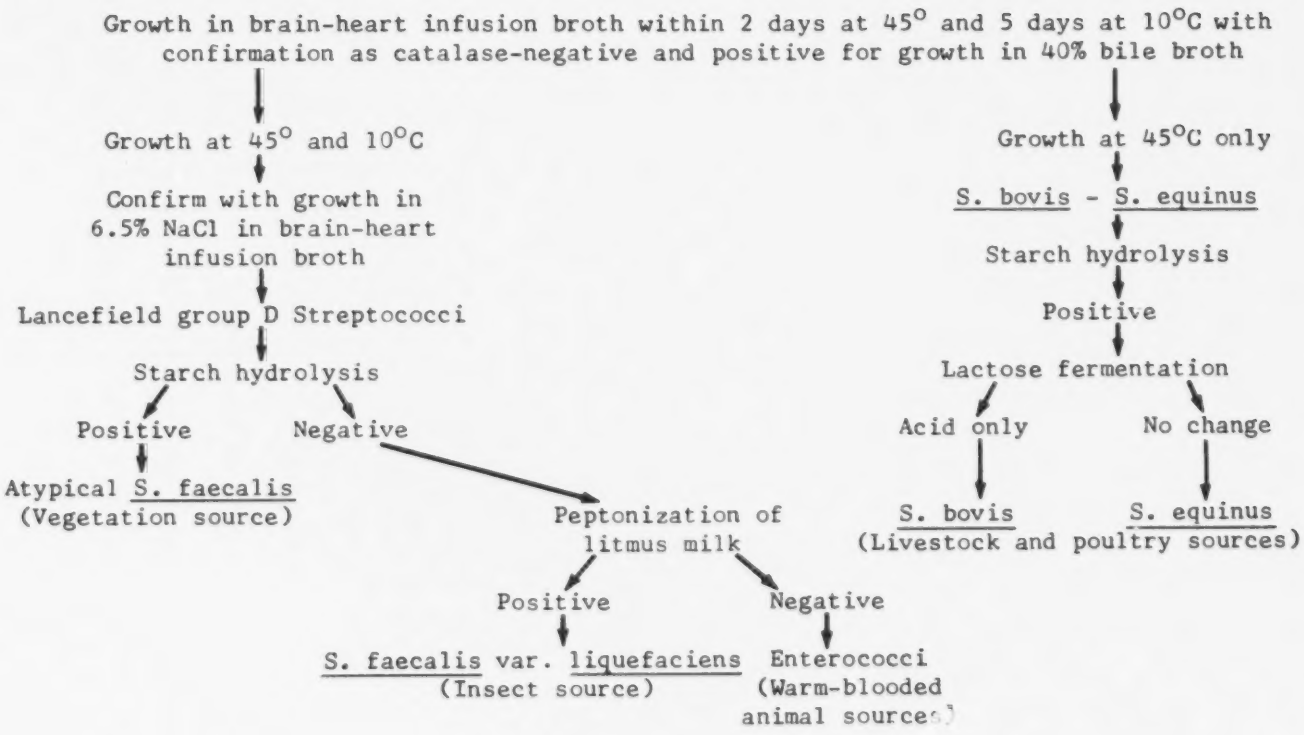
(b) a commensal type of growth on plants could be established —— as was noted with S. faecalis var. liquefaciens on beans, corn and cabbage plants;

(c) the tests for the detection and enumeration of the enterococci could have included the S. lactis organisms which are found on many plants. It is known that the AD-EVA procedure supports growth of S. lactis which are not normally associated with feces and the intestinal tract of man and animals.

The streptococci observed by Geldreich and co-authors hydrolyzed starch, were biochemically similar to S. faecalis and consequently were called atypical S. faecalis strains.[41] The prevalence of S. faecalis var. liquefaciens on plants and insects; its presence in the feces of cats, dogs and rodents; its presence in sanitary landfill,[42] which included normal domestic refuse, but not containing sludge or sewage has revealed the ubiquitous nature of this organism. These bacteria, therefore, are of a limited sanitary significance. The organism grows very well on KF and M-enterococcus agar, therefore, it will be included in the total fecal streptococcal count. From this MF (membrane filter) procedure using KF agar, identification can be made according to certain biochemical tests. The following scheme was proposed for separation of the variety S. liquefaciens, atypical S. faecalis and animal strains.[41]

SAMPLE

KF agar
Pink-red colonies


Growth in brain-heart infusion broth within 2 days at 45° and 5 days at 10°C with confirmation as catalase-negative and positive for growth in 40% bile broth
Growth at 45° and 10°C
Confirm with growth in 6.5% NaCl in brain-heart infusion broth
Lancefield group D Streptococci
Starch hydrolysis
Positive
Negative
Atypical S. faecalis (Vegetation source)
Peptonization of litmus milk
Positive
Negative
S. faecalis var. liquefaciens (Insect source)
Enterococci (Warm-blooded animal sources)
Growth at 45°C only
S. bovis - S. equinus
Starch hydrolysis
Positive
Lactose fermentation
Acid only
No change
S. bovis
S. equinus
(Livestock and poultry sources)

Note: A small percentage of unclassified strains (biotypes) will be found since fecal streptococci vary greatly in their biochemical reactions.

Schematic outline for identification of fecal streptococci

Survival

There are conflicting reports on the survival of fecal streptococci in soil. [43] S. faecalis inoculated into shallow pans of soil persisted much longer than did E. coli at room temperature and at 7°C. Under greenhouse conditions, S. faecalis succumbed more rapidly than E. coli. [20] Apparently, there are a number of factors such as pH, presence of organic matter, moisture and temperature which affect the survival of fecal streptococci in soil. For example, sludge added to seeded soil samples appeared to lengthen the survival time of S. faecalis.

Attempts have been made to study survival rates in soil, however, environmental conditions alter survival patterns considerably. It is impossible to take into account all of the components and complex interactions in soil therefore, survival data may only refer to and reflect conditions at the time of experimentation. For example, E. coli and S. faecalis [44] seeded at a protected hillside site survived twice as long as those present at an exposed lawn site during summer and autumn. During winter and spring, survival of both organisms at the hillside and lawn site were similar. However, during the summer the fecal streptococcus persisted slightly longer than the fecal coliform. In the autumn, there is little difference between the 2 organisms, however, in winter the fecal streptococcus survives much longer.

Increased persistence of the fecal streptococci during cold weather also occurs in storm water runoff. [45] S. faecalis, Aerobacter aerogenes,

Salmonella typhimurium and the fecal coliform persisted in higher densities and longer at 10°C than at 20°C. S. faecalis survived longer at both temperatures and at higher levels than did A. aerogenes, S. typhimurium or the fecal coliform. S. typhimurium survived slightly longer at 10°C than did A. aerogenes, but succumbed quicker than the fecal coliform during the first 11 days. The survival patterns of the fecal coliform and S. typhimurium therefore, were more similar than those of S. faecalis and S. typhimurium.

Of particular importance is the fecal streptococcus type distribution in storm water, rodents and pets.[45] Percentages of S. bovis and S. equinus group organisms were 17.1% in rodents and 32.0% in dogs. The organisms, however, could not be detected in the storm water samples analyzed. It was concluded that rapid die-off of these sensitive organisms contributed to the lack of isolation. As a result, recovery of such strains in storm water would indicate very recent soil contamination and subsequent transfer to the receiving stream by the storm water runoff.

The survival of enterococci in 1% raw sewage diluted with buffered water differed at 10°, 20° and 30°C.[46] With no noticeable lag phase, negative multiplication or aftergrowth, the death rate increased as the temperature increased. The average death rate for the enterococcus group was greater than the death rate for the coliform group at all 3 temperatures.

In an estuary, it was reported[46] that generally, there was a decrease of 50 to 85% of fecal streptococci within the first two days —— with complete die-off within 7 to 11 days. S. bovis disappeared within the first 24 hr. exposure. S. faecalis, S. faecium and S. faecalis var. liquefaciens exhibited similar death patterns, however, S. faecium appeared to be slightly more resistant. After 2 days, there was a die-off of 40%, 30% and 15% respectively for the 3 species. At the end of 7 days, S. faecalis and S. faecalis var. liquefaciens could not be detected, however, 1% of the original number of S. faecium were still present. With all of the enterococci tested in this particular estuary, where the water temperature was 18°C, multiplication or aftergrowth did not occur.

At seawater concentrations of 33 and 67%, similar death curves were noted for the enterococci.[47] With 100% seawater, a slight increase in the death rate was observed. Therefore, concentration of seawater seemed to show the least effect on enterococcal survival. This, however, was not so for the coliform and E. coli Type I organisms. E. coli Type I and coliform death rates were lower than that of the enterococci at 33 and 67% seawater concentration. No lag phases were observed for all three pollution indicators at the 67 and 100% seawater concentrations. The physiological resistivity of the enterococci to high salt or electrolyte concentration apparently does not significantly aid in increased survival in seawater.

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Detection

Selective media have proven most successful in the detection and enumeration of fecal streptococci. The use of sodium azide as the selective agent was probably the most significant development. Since the incorporation of this agent into selective bacteriological growth systems, numerous types of media appeared in the literature. In 1959 Croft[47] performed a comparative study on media for the detection of enterococci in water. The Azide Dextrose (AD)[48] and Ethyl Violet Azide (EVA) confirmatory of Litsky et. al.[49]; Buffered Azide Glucose Glycerol (BAGG)[50] with EVA confirmatory and M-enterococcus agar[51] were compared. AD-EVA and M-enterococcus were found to give the same order of productivity. BAGG broth gave a lower order of productivity in 70.4% of the samples tested. Slanetz et. al.[52], using a membrane filter (MF) technique with M-enterococcus agar received higher recoveries of enterococci than did the methods of Litsky et. al. and those of Winter and Sandholzer. Apparently, the M-enterococcus medium showed a high degree of selectivity for the enterococcus group organisms, because S. bovis, S. equinus and S. salivarius group organisms did not proliferate on this medium. However, it is difficult to conceive greater productivity with this type of selectivity, especially when S. bovis and S. equinus strains grow favorably in AD and EVA broths. It therefore, was not a surprise when Slanetz and Bartley[51] later reported a modification of the original M-enterococcus

medium. To give some idea of its productivity, ratios of enterococci to coliforms were given. For water, the ratio of enterococci to coliforms was 1.9:1; 1:1.7 for sewage samples; 1:1.6 for fecal samples from humans and 15:1 for fecal samples from animals. The claim was made that these ratios were the largest ever recorded for such samples.[51]

In 1960, Kenner and co-workers[53] described a liquid medium (KF) for the detection and enumeration of fecal streptococci. The medium could be used in a multiple tube procedure (MPN) or could be adapted to a membrane filter method. Results indicated that both types of procedures yielded higher recoveries than did M-enterococcus agar (MF), BAGG broth (MPN) or AD-EVA (MPN). The increased productivity, according to the authors, was due to the fact that a higher number of species could be isolated and a greater number of streptococcal species were able to grow on the KF medium. However, with the KF medium, the MF (membrane filter) procedure was recommended over the multiple tube procedure. Organisms in the form of colonies on the membrane, could be extracted quite easily for verification or species identification.

At the present time, for routine fecal streptococcal determinations in water using the MF procedure, KF and M-enterococcus agar have been employed. In a most recent comparison of the two media, Slanetz and Bartley[19] reported no significant difference in the recovery of fecal streptococci from fecal samples taken from human and hogs. On the

other hand, with sewage and river water samples, M-enterococcus agar exhibited significantly higher recoveries. The investigators also noted colonies on the membranes incubated on the KF saturated pads which were smaller and not as distinctly colored. This was attributed to the absence of agar in the KF medium. With seawater samples, only 28% of the colonies growing on KF membranes were reported as fecal streptococci, while M-enterococcus agar achieved a 98.5% confirmation. It must be noted at this point, that the number of samples used in the comparison by Slanetz was very small. On this basis, one wonders if the results obtained would still hold for a large number of samples.

A most exhaustive comparative study[20] with KF (MF) and M-enterococcus (MF) revealed greater productivity by the former procedure. Results were based on analysis of 401 estuary samples from Raritan Bay and 201 sewage treatment plant samples. The most likely explanation for the difference appears to lie in the fact that the 2 media actually do not enumerate the same types of streptococci. Depending upon the system used for the definition of streptococci occurring in feces, one medium may appear to be more selective and therefore produce densities of lower magnitudes. Confirmation data on colonies isolated from KF membranes indicate that KF may permit growth of a large number of streptococci and other organisms in seawater samples. Of 772 fecal

streptococcal-like colonies on KF membranes, 494 were sub-cultured for subsequent identification. Ninety-eight percent of the colonies proved to be fecal streptococci, while the remaining 2% were streptococci, but not of the fecal type. Ninety-one percent of the fecal strains belonged to the enterococcus group (Sherman) —— with the remainder falling in the biotype group. These results are not at all in accordance with those received by Slanetz[19] for seawater samples where only 24% of the colonies growing on KF membranes were fecal streptococci. Exact reasons for this low, reported confirmation rate are not known, however, it is strongly suspected that a lack of adequate sample numbers and/or a difference in the definition and classification of fecal streptococci constitute such results.

It has been noted[20] that great care must be exercised when using KF medium. Seawater samples processed using KF agar (MF) show a very high amount of background growth. This tends to interfere with discrete colony formation on the membrane. It also brings into play a number of colonies which approach the color characteristics of the fecal streptococci. Without the use of adequate lighting and magnification, brown-colored colonies can be mistaken for the deep-reddish types. The brown-colored colonies are not fecal streptococci. In this respect, M-enterococcus agar appears to be easier to work with because much of this background growth is eliminated. On occassion, very small pin-point, pink colonies have appeared on KF agar membranes. Normally, pink colonies

are included in the fecal streptococcus count. However, these colonies do not confirm as fecal streptococci. Approximately 50% of such colonies fail to grow upon initial transfer into BHI broth. A number of these type colonies, upon microscopic examination resembled yeast cells. On the basis of such observations, whenever such type colonies are encountered, confirmation must be performed before inclusion in the total fecal streptococcus count. Such pin-point, pink colonies were absent from M-enterococcus agar membranes.

Conclusion

The following points have been developed from the above literature survey:

1. The fecal streptococci that are of importance in sanitary microbiology and can serve effectively as indicators of fecal pollution are: S. faecalis, S. faecalis var. zymogenes, S. durans, S. bovis, S. equinus, S. salivarius and the S. faecalis biotype groups.

2. Organisms that should be eliminated from the group of fecal streptococcal indicators are: S. faecalis var. liquefaciens and typical S. faecalis strains that hydrolyze starch (atypical S. faecalis).

3. S. faecalis or enterococcus group organisms (Sherman) do not solely signify human pollution. These organisms are almost ubiquitous among warm-blooded animals and can also be found in association with cold-blooded animals.

4. Ratios of fecal coliforms to fecal streptococci are useful in detecting storm water and soil drainage effects. Ratios may also indicate the effects of wastes from meat packing houses and agricultural animal wastes. The ratio of fecal coliform to fecal streptococci for man is approximately 4.4 while that for other warm-blooded animals is 0.6 or less.[41] Therefore, ratios greater than 4:1 normally will indicate domestic waste. Ratios of less than 0.6 suggest livestock or farm animal wastes or storm water runoff which may have been contaminated by these waste materials. It must be remembered that ratios can be severely

effected when the pH of the water is less than 4.0 and above 9.0. Ratios are erroneous when, for example, the waste introduced into water has exceeded 24 hrs. travel downstream. Such farm or meat packing house waste normally containing S. bovis and/or S. equinus groups will have succumbed during the first 24 hr. exposure.

5. Neither KF nor M-enterococcus agar are ideal media for the detection and enumeration of fecal streptococci. Both media allow for the proliferation of S. faecalis var. liquefaciens and the atypical S. faecalis strains which may be associated with insects and vegetation. This necessitates confirmation of colonies from membranes when such organisms are suspected in the sample. M. Enterococcus agar appears to be too selective while KF agar frequently shows high background and aberrant, pink, pin-point fecal streptococcus-like colonies which do not grow upon subculture into BHI broth. Fecal streptococcus colonies on KF agar may show a wide variation of pink and purple shades while some deep reds begin to merge with brown non-fecal streptococcus colonies when seawater samples are being analyzed.

6. Serology appears to have some use in distinguishing the fecal streptococci. Further research, however, is needed to establish the feasibility of this approach. The true relationship of the S. faecalis biotypes (or sometimes called enterococcus biotype group organisms) to the enterococcus group species needs to be established.

7. Lack of uniformity exists in definition, classification and biochemical characterization which will identify species or sub-groups. Methodology used in the identification schema require standardization.

8. The fecal streptococci do, however, possess many characteristics which make them useful indicator organisms.

(a) Limited survival and lack of multiplication in water provide information on the recency of wastes entering the receiving stream.

(b) The animal specificity of S. bovis allows for its use in indicating non-human animal pollution in special studies.

(c) With the exception of the aberrant forms noted above, the fecal streptococci are derived from the intestinal tract of man and animals. Their presence therefore, signifies fecal or sewage pollution.

Research

It is felt, that although the fecal streptococci as indicators of fecal contamination possess inadequacies, the condition is not malignant. At the present time, investigators with a knowledge of the advantages and limitations and with the availability of competent microbiologists and technicians, can secure information presently not obtainable from coliform and fecal coliform data. A well planned university research grant program designed to develop a suitable recovery medium while investigating serological applications, species or sub-group relationships, classification and source definitions may retain a much needed tool for specific pollution investigations. One must recognize that the application of the fecal streptococci to pollution detection is quite recent. Experience with the fecal streptococci number about 10 years, while over 60 years of data on the coliform group of bacteria has been accumulated.

A good deal of information on the fecal streptococci has been developed through expensive and time consuming research. All of the information accumulated to date, whether negative or positive has contributed to a knowledge and better understanding of this diversified group of bacteria. It is the general consensus of research microbiologists in the field to build upon this information and to utilize it in the development of a reliable, accurate and descriptive indicator of fecal pollution. Once reliable information can be obtained on types, numbers and occurrence and antigenic relationships be established, this

group of bacteria can be readily applied to automatic monitoring systems. Antigen-antibody reactions coupled with fluorescein isothiocyanate and radioactive labeling has already shown promise. Fluorescent antibodies with very high degrees of labeling, achieved by attaching reactive "tails" to the antibodies and the use of labeled enzymes for the detection of biological agents are other approaches. Such principles are vital for rapid, sensitive automatic detection. Essential to this development, however, are basic segments of information:

1. Which of the streptococci are to be used in the detection system?

2. Based on survival patterns and physiological characteristics —— what levels of detection can we expect and therefore, what minimal detection limits do we need?

3. What is the significance of these numbers with respect to level of hazard?

4. What is the relationship of fecal streptococci to enteric pathogenic bacteria and viruses?

Answers to such questions must be made available before automatic monitoring becomes practical. Total coliforms do not realistically evaluate fecal contamination. The fecal coliforms consist of bacteria with wide serological characteristics therefore are not ideal for automatic monitoring which utilize the above principles. The fecal streptococci are the most eligible candidates for use in such a system. This, however, may never be realized until the basic and primary information outlined above is obtained.

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